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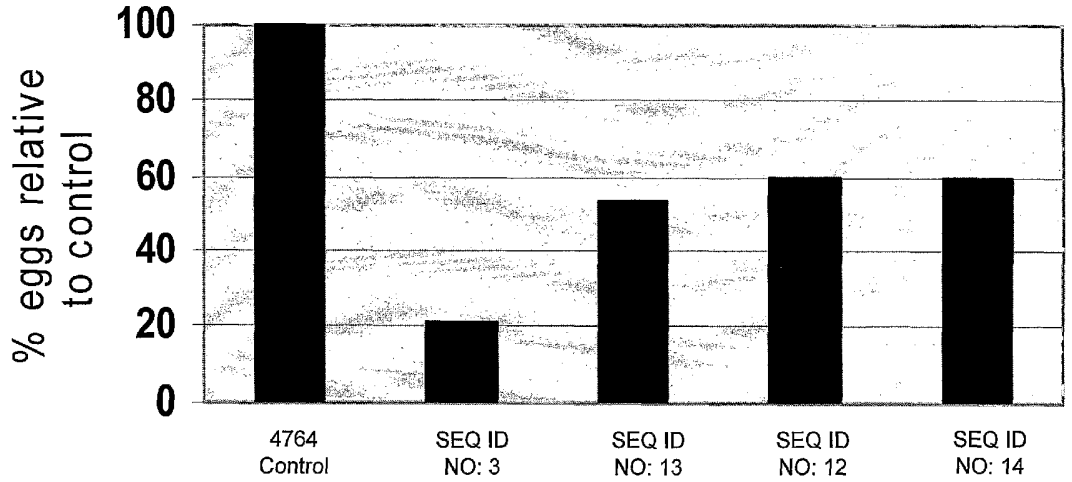
(54) Title: METHODS OF PROTECTING PLANTS FROM PATHOGENIC FUNGI AND NEMATODES



(57) Abstract: Methods for protecting a plant from a pathogen, particularly a pathogenic fungus or nematode, are provided. A method for enhancing pathogen resistance in a plant using the nucleotide sequences disclosed herein is further provided. The method comprises introducing into a plant an expression cassette comprising a promoter operably linked to a nucleotide sequence that encodes an antipathogenic polypeptide of the invention. Transformed plants, plant cells, seeds, and microorganisms comprising a nucleotide sequence that encodes an antipathogenic polypeptide of the embodiments, or variant or fragment thereof, are also disclosed.

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METHODS OF PROTECTING PLANTS FROM PATHOGENIC FUNGI AND NEMATODES

FIELD OF THE INVENTION

The present invention relates to methods of protecting plants from pathogens through the use of polypeptides having antifungal and/or nematicidal activity and the nucleic acid sequences that encode them. Methods of the invention utilize these polypeptides and nucleic acid sequences to control plant pathogens and to increase pathogen resistance in plants. Transgenic plants and seeds are also included.

BACKGROUND OF THE INVENTION

Disease in plants results from biotic and abiotic causes. Biotic causes include, but are not limited to, fungi and nematodes. A host of cellular processes enables plants to defend themselves from disease caused by pathogenic agents. These processes apparently form an integrated set of resistance mechanisms that is activated by initial infection and then limits further spread of the invading pathogenic organism.

Subsequent to recognition of a plant pathogen, plants can activate an array of biochemical responses. Generally, the plant responds by inducing several local responses in the cells immediately surrounding the infection site. The most common resistance response observed in both nonhost and race-specific interactions is termed the "hypersensitive response" (HR). In the hypersensitive response, cells contacted by the pathogen, and often neighboring cells, rapidly collapse and dry in a necrotic fleck. Other responses include the deposition of callose, the physical thickening of cell walls by lignification, and the synthesis of various antibiotic small molecules and proteins. Genetic factors in both the host and the pathogen determine the specificity of these local responses, which can be very effective in limiting the spread of infection.

Incidence of plant diseases has traditionally been controlled by agronomic practices that include crop rotation, the use of agrochemicals, and conventional breeding techniques. The use of chemicals to control plant pathogens, however, increases costs to farmers and causes harmful effects on the ecosystem. Consumers and government regulators alike are becoming increasingly concerned with the environmental hazards associated with the production and use of synthetic

agrochemicals for protecting plants from pathogens. Because of such concerns, regulators have banned or limited the use of some of the most hazardous chemicals. The incidence of fungal diseases has been controlled to some extent by breeding resistant crops. Traditional breeding methods, however, are time-consuming and require continuous effort to maintain disease resistance as pathogens evolve. See, for example, Grover and Gowthaman (2003) *Curr. Sci.* 84:330-340. Thus, there is a significant need for novel alternatives for the control of plant pathogens that possess a lower risk of pollution and environmental hazards than is characteristic of traditional agrochemical-based methods and that are less cumbersome than conventional breeding techniques.

Recently, agricultural scientists have developed crop plants with enhanced pathogen resistance by genetically engineering plants to express antipathogenic proteins. For example, potatoes and tobacco plants genetically engineered to produce an antifungal endochitinase protein were shown to exhibit increased resistance to foliar and soil-borne fungal pathogens. See Lorito *et al.* (1998) *Proc. Natl. Acad. Sci.* 95:7860-7865. Moreover, transgenic barley that is resistant to the stem rust fungus has also been developed. See Horvath *et al.* (2003) *Proc. Natl. Acad. Sci.* 100:364-369. A continuing effort to identify antipathogenic agents and to genetically engineer disease-resistant plants is underway.

Nematode infection is a significant problem in the farming of many agriculturally significant crops. For example, soybean cyst nematode *(Heterodera glycines)* is a widespread pathogen that is believed to be responsible for yield losses in soybeans, estimated to be in excess of $1 billion per year in North America. Such damage is the result of the stunting of the soybean plant caused by the cyst nematode. The stunted plants have smaller root systems, show symptoms of mineral deficiencies in their leaves, and wilt easily. Other pathogenic nematodes of significance to agriculture include the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida,* which are key pathogens of the potato, the cotton cyst nematode, *Meloidogyne incognita*, and the beet cyst nematode *Heterodera schachtii*, which is a major problem for sugar beet growers in Europe and the United States.

The primary method of controlling nematodes has been through the application of highly toxic chemical compounds. The widespread use of chemical compounds poses many problems with regard to the environment because of the non-selectivity of

the compounds and the development of insect resistance to the chemicals. Nematicides such as Aldicarb and its breakdown products are known to be highly toxic to mammals. As a result, government restrictions have been imposed on the use of these chemicals. The most widely used nematicide, methyl bromide, is scheduled to be soon retired from use, and at present, there is no promising candidate to replace this treatment. Thus, there is a great need for effective, non-chemical methods and compositions for nematode control.

Various approaches to pathogen control have been tried including the use of biological organisms which are typically "natural predators" of the species sought to be controlled. Such predators may include other insects, fungi, and bacteria such as *Bacillus thuringiensis.* Alternatively, large colonies of insect pests have been raised in captivity, sterilized and released into the environment in the hope that mating between the sterilized insects and fecund wild insects will decrease the insect population. While these approaches have had some success, they entail considerable expense and present several major difficulties. For example, it is difficult both to apply biological organisms to large areas and to cause such living organisms to remain in the treated area or on the treated plant species for an extended time. Predator insects can migrate and fungi or bacteria can be washed off of a plant or removed from a treated area by rain. Consequently, while the use of such biological controls has desirable characteristics and has met with some success, in practice these methods have not achieved the goal of controlling pathogen damage to crops.

Advances in biotechnology have presented new opportunities for pathogen control through genetic engineering. In particular, advances in plant genetics coupled with the identification of naturally-occurring plant defensive compounds or agents offer the opportunity to create transgenic crop plants capable of producing such defensive agents and thereby protect the plants against disease.

One promising method for nematode control is the production of transgenic plants that are resistant to nematode infection and reproduction. For example, with the use of nematode-inducible promoters, plants can be genetically altered to express nematicidal proteins in response to exposure to nematodes. See, for example, U.S. Patent No. 6,252,138, herein incorporated by reference. Similarly, with the use of proteins having a deleterious effect on nematodes, plants can be genetically altered to express such deleterious proteins in response to nematode attack.

Thus, in light of the significant impact of plant pathogens, particularly fungal and nematode pathogens, on the yield and quality of crops, new methods for protecting plants from such pathogens are needed.

BRIEF SUMMARY OF THE INVENTION

The embodiments of the invention provide transgenic plants with enhanced resistance to pathogens, each plant comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence at least 95% identical to SEQ ID NOs: 1, 2, 4, 5, 7, 8, 10 or 11, wherein said plant has improved pathogen resistance to at least one pathogen selected from the group consisting of plant pathogenic fungi and nematodes. The plant may be a monocot or a dicot. Seeds of such transgenic plants are also provided for. Similarly, the embodiments provide monocot or dicot transgenic plants and seeds with enhanced resistance to pathogens wherein the plant comprises a polynucleotide sequence at least 95% identical to SEQ ID NOs: 3, 6, 9, 12, 13, or 14, wherein said plant has improved pathogen resistance to at least one pathogen selected from the group consisting of plant pathogenic fungi and nematodes. The polypeptides expressed in the transgenic plants may or may not comprise a signal sequence.

The embodiments of the invention also provide methods of enhancing resistance of a plant to a pathogen, the methods comprising introducing into a plant cell an expression cassette comprising a nucleotide sequence operably linked to a promoter, wherein the nucleotide sequence has at least 95% identity to SEQ ID NOs: 3, 6, 9, 12, 13 or 14, or wherein the nucleotide sequence encodes a polypeptide comprising an amino acid sequence identical or substantially identical to SEQ ID NOs: 1, 2, 4, 5, 7, 8, 10 or 11, and wherein the polypeptide has activity against at least one pathogen selected from the group consisting of plant pathogenic fungi and nematodes. The plant cell is used to regenerate a transformed plant wherein the level of pathogen resistance in the transformed plant is increased in comparison to a plant that does not comprise the expression cassette. The polypeptides of these embodiments may or may not comprise a signal sequence.

The promoters used in the expression cassettes of the embodiments are selected from the group consisting of constitutive, tissue-specific, root-specific, inducible and pathogen-inducible promoters. In some embodiments, the

polypeptide with activity against plant pathogens comprises a signal sequence. In some embodiments, the polypeptide lacks a signal sequence. In some embodiments, the signal sequence is a secretion signal sequence, while in others it is an organelle and/or plastid signal sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts results from a *Meloidogyne incognita* infection assay in Astragalus hairy roots.

Figure 2 depicts results from a *Meloidogyne incognita* infection assay in transgenic tobacco plants.

DETAILED DESCRIPTION OF THE INVENTION

Embodiments of the invention provide compositions and methods directed to enhancing pathogen resistance, particularly nematode and fungal resistance, in plants. The embodiments provide polynucleotides encoding amino acid sequences for nematicidal and antifungal polypeptides. Specifically, the embodiments provide nematicidal and antifungal polypeptides having the amino acid sequences set forth in SEQ ID NOs: 1, 2, 4, 5, 7, 8, 10 and 11 and variants and fragments thereof. Isolated nucleic acid molecules, and variants and fragments thereof, comprising nucleotide sequences that encode the amino acid sequences shown in SEQ ID NOs: 1, 2, 4, 5, 7, 8, 10 and 11 are further provided.

Nucleotide sequences that encode the polypeptides of SEQ ID NOs: 1, 2, 4, 5, 7, 8, 10 and 11 are provided. These nucleotide sequences are set forth in SEQ ID NOs:3, 6, 9, 12, 13, and 14. Some of these nucleotide sequences have been optimized for expression in E. coli. Plants, plant cells, seeds, and microorganisms comprising a nucleotide sequence that encodes a nematicidal and/or antifungal polypeptide of the embodiments are also disclosed herein. Nematicidal and antifungal compositions comprising an isolated nematicidal polypeptide or antifungal polypeptide or a microorganism that expresses a polypeptide of the embodiments are further provided. The compositions of the embodiments find use in generating nematode-resistant and fungal-resistant plants and in protecting plants from nematodes and fungi.

The polypeptides disclosed herein display activity against nematodes, such as, for example, *Caenorhabditis elegans* and *Meloidogyne incognita*. The

polypeptides disclosed herein also display antifungal activity against plant pathogenic fungi, such as, for example, *Fusarium oxysporum* and *Verticillium dahliae*. The species of origin of these nematicidal and antifungal polypeptides are plant species. In particular, the source of the polypeptides of SEQ ID NOs:1, 2, 7, and 8 is *Arabidopsis thaliana*. The source of the polypeptides of SEQ ID NOs: 4 and 5 is *Picea mariana* (black spruce), and the source of the polypeptides of SEQ ID NOs: 10 and 11 is *Petunia x hybrida*.

"Nematicidal compositions" or "nematicidal polypeptides" is intended to mean that the compositions or polypeptides of the embodiments have nematicidal activity and thus are capable of suppressing, controlling, and/or killing the invading nematode. "Antifungal compositions" or "antifungal polypeptides" is intended to mean that the compositions or polypeptides of the embodiments have antifungal activity and thus are capable of suppressing, controlling, and/or killing the invading fungus. A nematicidal or antifungal polypeptide of the embodiments will reduce the disease symptoms resulting from nematode or fungal challenge by at least about 5% to about 50%, at least about 10% to about 60%, at least about 30% to about 70%, at least about 40% to about 80%, or at least about 50% to about 90% or greater. Hence, the methods of the embodiments can be utilized to protect plants from fungi and nematodes.

The polynucleotides and polypeptides of the embodiments find use in methods for inducing pathogen resistance in a plant. Accordingly, the compositions and methods disclosed herein are useful in protecting plants against nematodes and fungi. "Pathogen resistance" is intended to mean that the plant avoids the disease symptoms that are the outcome of plant-nematode interactions or plant-fungus interactions. A plant with "improved pathogen resistance" or "enhanced pathogen resistance" is intended to mean that a plant, which has been transformed with a nucleic acid molecule of the embodiments, and which is expressing a polypeptide of the embodiments, exhibits a level of resistance or tolerance to a fungal or nematode pathogen that is increased in comparison to a plant that does not comprise said nucleic acid molecule, such as a wild type plant. That is, nematodes or fungi are prevented from causing plant disease and the associated disease symptoms in the transformed plant, or alternatively, the disease symptoms caused by the nematode or fungus are minimized or lessened, such as, for example, the reduction of stress and associated yield loss. Resistance may vary

from a slight increase in tolerance to the effects of the pathogen to total resistance such that the plant is unaffected by the presence of the pathogen. An increased level of resistance against a particular fungus or nematode or against a wider spectrum of fungi or nematodes may both constitute antifungal and nematicidal activity or improved nematode or fungus resistance. The plants of the embodiments exhibit an improvement of at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or up to 100% improvement compared to an untransformed plant. Such improvement may be measured by any suitable means known in the art, such as, but not limited to, counting cysts or eggs of nematodes, counting fungal lesions on plants, measuring fungal biomass, comparing plant yields, and other methods described in the following paragraphs.

Assays that measure antipathogenic activity are commonly known in the art, as are methods to quantitate disease resistance in plants following pathogen infection. See, for example, U.S. Patent No. 5,614,395, herein incorporated by reference. Such techniques include, measuring over time, the average lesion diameter, the pathogen biomass, and the overall percentage of decayed plant tissues. For example, a plant either expressing an antipathogenic polypeptide or having an antipathogenic composition applied to its surface shows a decrease in tissue necrosis (*i.e.,* lesion diameter) or a decrease in plant death following pathogen challenge when compared to a control plant that was not exposed to the antipathogenic composition. Alternatively, antipathogenic activity can be measured by a decrease in pathogen biomass. For example, a plant expressing an antipathogenic polypeptide or exposed to an antipathogenic composition is challenged with a pathogen of interest. Over time, tissue samples from the pathogen-inoculated tissues are obtained and RNA is extracted. The percent of a specific pathogen RNA transcript relative to the level of a plant specific transcript allows the level of pathogen biomass to be determined. See, for example, Thomma *et al.* (1998) *Plant Biology* 95:15107-15111, herein incorporated by reference.

Furthermore, *in vitro* antipathogenic assays include, for example, the addition of varying concentrations of the antipathogenic composition to paper disks and placing the disks on agar containing a suspension of the pathogen of interest. Following incubation, clear inhibition zones develop around the discs that contain

an effective concentration of the antipathogenic polypeptide (Liu *et al.* (1994) *Plant Biology* 91:1888-1892, herein incorporated by reference). Additionally, microspectrophotometrical analysis can be used to measure the *in vitro* antipathogenic properties of a composition (Hu *et al.* (1997) *Plant Mol. Biol.* 34:949-959 and Cammue *et al.* (1992) *J. Biol. Chem.* 267: 2228-2233, both of which are herein incorporated by reference). Assays that specifically measure antifungal activity are also well known in the art. See, for example, Duvick *et al.* (1992) *J. Biol. Chem.* 267:18814-18820; Lacadena *et al.* (1995) *Arch. Biochem. Biophys.* 324:273-281; Xu *et al.* (1997) *Plant Mol. Biol.* 34: 949-959; Lee *et al.* (1999) *Biochem. Biophys. Res. Comm.* 263:646-651; Vila *et al.* (2001) *Mol. Plant Microbe Interact.* 14:1327-1331; Moreno *et al.* (2003) *Phytpathol.* 93:1344-1353; Kaiserer *et al.* (2003) *Arch. Microbiol.* 180:204-210; and U.S. Patent No. 6,015,941.

Also contemplated are antipathogenic assays directed at nematode pathogens. Such assays are known to the skilled artisan, and may include assays directed at specific characteristics of nematode pathogen infections, such as assays directed at nematode feeding site formation. Such assays include those disclosed in U.S. Patent Nos. 6,008,436; and 6,252,138; herein incorporated by reference.

The embodiments disclose plants transformed with nucleic acid molecules that encode nematicidal and antifungal proteins. The compositions find use in methods for inducing pathogen resistance in a plant and for protecting a plant from a fungus or a nematode. One of skill in the art will appreciate that the compositions and methods disclosed herein can be used in combination with other compositions and methods available in the art for protecting plants from pathogen attack.

In particular aspects, methods for inducing nematode or fungal resistance in a plant comprise introducing into a plant at least one expression cassette, wherein the expression cassette comprises a nucleotide sequence encoding a nematicidal or antifungal polypeptide of the embodiments operably linked to a promoter that drives expression in the plant. The plant expresses the polypeptide, thereby exposing the nematode or fungus to the polypeptide at the site of attack. Expression of a polypeptide of the embodiments may be targeted to specific plant tissues where nematode or fungal resistance is particularly important, such as, for example, roots, leaves, or stems. Such tissue-preferred expression may be accomplished by root-preferred, leaf-preferred, vascular tissue-preferred, stalk-

preferred, or seed-preferred promoters. Moreover, the polypeptides of the embodiments may also be targeted to specific subcellular locations within a plant cell or, alternatively, secreted from the cell, as described herein below.

Just as expression of a polypeptide of the embodiments may be targeted to specific plant tissues or cell types through the use of appropriate promoters, it may also be targeted to different locations within the cell through the use of targeting information or "targeting labels." Unlike the promoter, which acts at the transcriptional level, such targeting information is part of the initial translation product. Depending on the mode of infection of the pathogen or the metabolic function of the tissue or cell type, the location of the protein in different compartments of the cell may make it more efficacious against a given pathogen or make it interfere less with the functions of the cell. For example, one may produce a protein preceded by a signal peptide, which directs the translation product into the endoplasmic reticulum, by including in the construct (i.e. expression cassette) sequences encoding a signal peptide (such sequences may also be called the "signal sequence"). The signal sequence used could be, for example, one associated with the gene encoding the polypeptide, or it may be taken from another gene.

There are many signal peptides described in the literature, and they are largely interchangeable (Raikhel and Chrispeels, "Protein sorting and vesicle traffic" in Buchanan *et al.*, eds, (2000) *Biochemistry and Molecular Biology of Plants* (American Society of Plant Physiologists, Rockville, MD), herein incorporated by reference). The addition of a signal peptide will result in the translation product entering the endoplasmic reticulum (in the process of which the signal peptide itself is removed from the polypeptide), but the final intracellular location of the protein depends on other factors, which may be manipulated to result in localization most appropriate for the pathogen and cell type. The default pathway, that is, the pathway taken by the polypeptide if no other targeting labels are included, results in secretion of the polypeptide across the cell membrane (Raikhel and Chrispeels, *supra*) into the apoplast. The apoplast is the region outside the plasma membrane system and includes cell walls, intercellular spaces, and the xylem vessels that form a continuous, permeable system through which water and solutes may move. This will often be a suitable location.

Other pathogens may be more effectively combated by locating the peptide within the cell rather than outside the cell membrane. This can be accomplished, for example, by adding an endoplasmic reticulum retention signal encoding sequence to the sequence of the gene. Methods and sequences for doing this are described in Raikhel and Chrispeels, *supra*; for example, adding sequences encoding the amino acids K, D, E and L in that order, or variations thereof described in the literature, to the end of the protein coding portion of the polypeptide will accomplish this. ER retention sequences are well known in the art. See, for example, Denecke *et al.* (1992). *EMBO J.* 11:2345-2355; Wandelt *et al.* (1992) *Plant J.* 2:181-192; Denecke *et al.* (1993) *J. Exp. Bot.* 44:213-221; Vitale *et al.* (1993) *J. Exp. Bot.* 44:1417-1444; Gomord *et al.* (1996) *Plant Physiol. Biochem.* 34:165-181; Lehmann *et al.* (2001) *Plant Physiol.* 127 (2): 436–449.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues (*e.g.*, peptide nucleic acids) having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides.

The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Polypeptides of the embodiments can be produced either from a nucleic acid molecule disclosed herein, or by the use of standard molecular biology techniques. For example, a truncated protein of the embodiments can be produced by expression of a recombinant nucleic acid molecule of the embodiments in an appropriate host cell, or alternatively by a combination of *ex vivo* procedures, such as protease digestion and purification.

As used herein, the terms "encoding" or "encoded" when used in the context of a specified nucleic acid molecule mean that the nucleic acid molecule comprises the requisite information to direct translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid molecule encoding a protein may comprise non-translated sequences (*e.g.*, introns) within translated regions of the nucleic acid

sequence or may lack such intervening non-translated sequences (*e.g.*, as in cDNA).

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

The embodiments encompass methods of using isolated or substantially purified polynucleotide or protein compositions. An "isolated" or "purified" polynucleotide or protein, or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the polynucleotide or protein as found in its naturally occurring environment. Thus, an isolated or purified polynucleotide or protein is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Optimally, an "isolated" polynucleotide is free of sequences (optimally protein encoding sequences) that naturally flank the polynucleotide (i.e., sequences located at the 5' and 3' ends of the polynucleotide) in the genomic DNA of the organism from which the polynucleotide is derived. For example, in various embodiments, the isolated polynucleotide can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequence that naturally flank the polynucleotide in genomic DNA of the cell from which the polynucleotide is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of contaminating protein. When the protein of the embodiments or biologically active portion thereof is recombinantly produced, optimally culture medium represents less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the embodiments. "Fragment" is

intended to mean a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein and hence have antipathogenic activity, more particularly nematicidal or antifungal activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the polypeptides of the embodiments.

A fragment of a nucleotide sequence that encodes a biologically active portion of an antipathogenic polypeptide of the embodiments will encode at least 15, 25, 30, 40, or 50 contiguous amino acids, or up to the total number of amino acids present in a full-length antifungal polypeptide of the embodiments (for example, 66 amino acids for SEQ ID NO:1). Fragments of a nucleotide sequence that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of an antipathogenic protein.

As used herein, "full-length sequence" in reference to a specified polynucleotide means having the entire nucleic acid sequence of a native sequence. "Native sequence" is intended to mean an endogenous sequence, *i.e.*, a non-engineered sequence found in an organism's genome.

Thus, a fragment of a nucleotide sequence of the embodiments may encode a biologically active portion of an antipathogenic polypeptide, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an antipathogenic polypeptide can be prepared by isolating a portion of one of the nucleotide sequences of the embodiments, expressing the encoded portion of the antipathogenic protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the nematicidal or antifungal protein. Nucleic acid molecules that are fragments of a nucleotide sequence of the embodiments comprise at least 15, 20, 50, 75, 100, or 150 contiguous nucleotides, or up to the number of nucleotides present in a full-length nucleotide sequence disclosed herein.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a deletion and/or addition of one or more nucleotides at one or more internal sites within the native polynucleotide and/or a

substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. One of skill in the art will recognize that variants of the nucleic acid sequences of the embodiments will be constructed such that the open reading frame is maintained. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the antipathogenic polypeptides of the embodiments. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotide, such as those generated, for example, by using site-directed mutagenesis but which still encode an antipathogenic protein of the embodiments. Generally, variants of a particular polynucleotide of the embodiments will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein.

Variants of a particular polynucleotide of the embodiments (i.e., the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Thus, for example, an isolated polynucleotide that encodes a polypeptide with a given percent sequence identity to the polypeptides of SEQ ID NOs: 1, 3, 5, 7, and 9 are disclosed. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides of the embodiments is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

"Variant" protein is intended to mean a protein derived from the native protein by deletion or addition of one or more amino acids at one or more internal sites in the native protein and/or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the embodiments are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, antipathogenic, particularly nematicidal or antifungal activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native antipathogenic protein of the embodiments will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a protein of the embodiments may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The proteins of the embodiments may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants and fragments of the antipathogenic proteins can be prepared by mutations in the DNA. Methods for mutagenesis and polynucleotide alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be optimal.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an

amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, in a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(*see, e.g.*, Creighton, *Proteins* (1984)).

Thus, the genes and polynucleotides of the embodiments include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the embodiments encompass naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired antipathogenic, particularly nematicidal or antifungal, activity. Obviously, the

mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and optimally will not create complementary regions that could produce secondary mRNA structure. See, EP Patent No. 0075444.

In nature, some polypeptides are produced as complex precursors which, in addition to targeting labels such as the signal peptides discussed elsewhere in this application, also contain other fragments of peptides which are removed (processed) at some point during protein maturation, resulting in a mature form of the polypeptide that is different from the primary translation product (aside from the removal of the signal peptide). "Mature protein" refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor protein" or "prepropeptide" or "preproprotein" all refer to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may include, but are not limited to, intracellular localization signals. "Pre" in this nomenclature generally refers to the signal peptide. The form of the translation product with only the signal peptide removed but no further processing yet is called a "propeptide" or "proprotein." The fragments or segments to be removed may themselves also be referred to as "propeptides." A proprotein or propeptide thus has had the signal peptide removed, but contains propeptides (here referring to propeptide segments) and the portions that will make up the mature protein. The skilled artisan is able to determine, depending on the species in which the proteins are being expressed and the desired intracellular location, if higher expression levels might be obtained by using a gene construct encoding just the mature form of the protein, the mature form with a signal peptide, or the proprotein (i.e., a form including propeptides) with a signal peptide. For optimal expression in plants or fungi, the pre- and propeptide sequences may be needed. The propeptide segments may play a role in aiding correct peptide folding.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by assays that measure antipathogenic activity such as, for example, antifungal plate assays and other methods described

elsewhere in this disclosure. See, for example, Duvick *et al.* (1992) *J. Biol. Chem.* 267:18841-18820, herein incorporated by reference.

Variant polynucleotides and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different antipathogenic protein coding sequences can be manipulated to create a new antipathogenic protein possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the antipathogenic protein gene of the embodiments and other known antipathogenic protein genes to obtain a new gene coding for a protein with an improved property of interest, such as increased antifungal or nematicidal activity. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Crameri *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA* 94:4504-4509; Crameri *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The polynucleotides of the embodiments can be used to isolate corresponding sequences from other organisms, particularly other plants. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire sequences set forth herein or to variants and fragments thereof are encompassed by the embodiments. Such sequences include sequences that are orthologs of the disclosed sequences. "Orthologs" is intended to mean genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share at least 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. Functions of orthologs are often highly conserved among species. Thus, isolated polynucleotides that encode for an antipathogenic,

particularly antifungal or nematicidal protein and which hybridize under stringent conditions to the sequences disclosed herein, or to variants or fragments thereof, are encompassed by the embodiments.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any organism of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to; methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known polynucleotide is used as a probe that selectively hybridizes to other corresponding polynucleotides present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the polynucleotides of the embodiments. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire polynucleotide disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding polynucleotides and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among antipathogenic polynucleotide sequences and are optimally at least about 10 nucleotides in length, and most optimally at least about 20 nucleotides in length. Such probes may be used to amplify corresponding polynucleotides from a

chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al. supra*).

Hybridization of such sequences may be carried out under stringent conditions. "Stringent conditions" or "stringent hybridization conditions" is intended to mean conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optimally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 °C for short probes (e.g., 10 to 50 nucleotides) and at least about 60 °C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37 °C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55 °C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to 60 °C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a final wash in 0.1X SSC at 60 to 65 °C for at least 30 minutes. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours. The duration of the wash time will be at least a length of time sufficient to reach equilibrium.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the thermal melting point (T_m) can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: T_m = 81.5°C + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with ≥90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the T_m for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the T_m; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the T_m; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the T_m. Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is optimal to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acid sequences is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al. supra.*

The following terms are used to describe the sequence relationships between two or more polynucleotides or polypeptides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", and, (d) "percentage of sequence identity."

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two polynucleotides. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local alignment algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 872264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, California, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90;

Huang *et al.* (1992) *CABIOS* 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the embodiments. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the embodiments. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using Gap Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using Gap Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. "Equivalent program" is intended to mean any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of

matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the GCG Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the Quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the GCG Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature

of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

The use of the term "polynucleotide" is not intended to limit the embodiments to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides, can comprise ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The polynucleotides of the embodiments also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, and the like.

In some embodiments, expression cassettes comprising a promoter operably linked to a heterologous nucleotide sequence of the embodiments that encodes an antipathogenic polypeptide are further provided. The expression cassettes of the embodiments find use in generating transformed plants, plant cells, and microorganisms and in practicing the methods for inducing pathogen resistance disclosed herein. The expression cassette will include 5' and 3'

regulatory sequences operably linked to a polynucleotide of the embodiments. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide of interest and a regulatory sequence (i.e., a promoter) is a functional link that allows for expression of the polynucleotide of interest. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, by operably linked it is intended that the coding regions are in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of the polynucleotide that encodes an antipathogenic polypeptide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional initiation region (i.e., a promoter), translational initiation region, a polynucleotide of the embodiments, a translational termination region and, optionally, a transcriptional termination region functional in the host organism. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the polynucleotide of the embodiments may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the polynucleotide of the embodiments may be heterologous to the host cell or to each other. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide.

The optionally included termination region may be native with the transcriptional initiation region, may be native with the operably linked polynucleotide of interest, may be native with the plant host, or may be derived from

another source (i.e., foreign or heterologous) to the promoter, the polynucleotide of interest, the host, or any combination thereof. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639. In particular embodiments, the potato protease inhibitor II gene (PinII) terminator is used. See, for example, Keil *et al.* (1986) *Nucl. Acids Res.* 14:5641-5650; and An *et al.* (1989) *Plant Cell* 1:115-122, herein incorporated by reference in their entirety.

Where appropriate, the polynucleotides may be optimized for increased expression in the transformed organism. For example, the polynucleotides can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie *et al.* (1995) *Gene* 165(2):233-238), MDMV leader (Maize Dwarf Mosaic Virus), and human immunoglobulin heavy-chain binding

protein (BiP) (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, glyphosate and 2,4-dichlorophenoxyacetate (2,4-D). Additional selectable markers include phenotypic markers such as β-galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su et *al.* (2004) *Biotechnol Bioeng 85:*610-9 and Fetter *et al.* (2004) *Plant Cell 16:*215-28), cyan florescent protein (CYP) (Bolte *et al.* (2004) *J. Cell Science 117*:943-54 and Kato *et al.* (2002) *Plant Physiol 129*:913-42), and yellow florescent protein (PhiYFP™ from Evrogen, see, Bolte *et al.* (2004) *J. Cell Science 117*:943-54). For additional selectable markers, see generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University

of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334:721-724, and WO 02/36782. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the embodiments.

The term "promoter" refers to regions or sequence located upstream and/or downstream from the start of transcription that are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. Promoters include nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

A number of promoters can be used in the practice of the embodiments, including the native promoter of the polynucleotide sequence of interest. The promoters can be selected based on the desired outcome. A wide range of plant promoters are discussed in the recent review of Potenza *et al.* (2004) *In Vitro Cell Dev Biol – Plant 40:1-22*, herein incorporated by reference. For example, the nucleic acid molecules can be combined with constitutive, tissue-preferred, pathogen-inducible, or other promoters for expression in plants. Such constitutive

promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those disclosed in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611.

Generally, it will be beneficial to express the gene from an inducible promoter, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. See also WO 99/43819, herein incorporated by reference.

Of interest are promoters that result in expression of a protein locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. A further example is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium verticillioides* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200).

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the embodiments. Such wound-inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996)

Nature Biotechnology 14:494-498); wun1 and wun2, U.S. Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); systemin (McGurl *et al.* (1992) *Science* 225:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol.* 22:783-792; Eckelkamp *et al.* (1993) *FEBS Letters* 323:73-76); MPI gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like, herein incorporated by reference.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced expression of the antipathogenic polypeptides of the embodiments within a particular plant tissue. For example, a tissue-preferred promoter may be used to express a nematicidal or antifungal polypeptide in a plant tissue where disease resistance is particularly important, such as, for example, the roots or the leaves. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138;

Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Vascular tissue-preferred promoters are known in the art and include those promoters that selectively drive protein expression in, for example, xylem and phloem tissue. Vascular tissue-preferred promoters include, but are not limited to, the *Prunus serotina* prunasin hydrolase gene promoter (see, e.g., International Publication No. WO 03/006651), and also those found in US Patent Application Serial No. 10/109,488.

Stalk-preferred promoters may be used to drive expression of an antipathogenic polypeptide of the embodiments. Exemplary stalk-preferred promoters include the maize MS8-15 gene promoter (see, for example, U.S. Patent No. 5,986,174 and International Publication No. WO 98/00533), and those found in Graham *et al.* (1997) *Plant Mol Biol* 33(4): 729-735.

Leaf-preferred promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). Additional

root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase) (see WO 00/11177 and U.S. Patent No. 6,225,529; herein incorporated by reference). Gamma-zein is a preferred endosperm-specific promoter. Glob-1 is a preferred embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc. See also WO 00/12733, where seed-preferred promoters from *end1* and *end2* genes are disclosed; herein incorporated by reference.

In certain embodiments the nucleic acid sequences of the embodiments can be stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired phenotype. For example, the polynucleotides of the embodiments may be stacked with any other polynucleotides of the embodiments, such as any combination of SEQ ID NOS: 3, 6, 9, 12, 13, or 14, or with other nematicidal or antifungal genes and the like. The combinations generated can also include multiple copies of any one of the polynucleotides of interest. The polynucleotides of the embodiments can also be stacked with any other gene or combination of genes to produce plants with a variety of desired trait combinations including but not limited to traits desirable for animal feed such as high oil genes (*e.g.*, U.S. Patent No. 6,232,529); balanced amino acids (*e.g.* hordothionins (U.S. Patent Nos. 5,990,389; 5,885,801; 5,885,802; and 5,703,409); barley high lysine (Williamson *et al.* (1987) *Eur. J. Biochem.* 165:99-106; and WO 98/20122); and high methionine proteins (Pedersen *et al.* (1986) *J. Biol. Chem.* 261:6279; Kirihara *et al.* (1988) *Gene* 71:359; and Musumura *et al.* (1989) *Plant Mol. Biol.* 12: 123));

increased digestibility (*e.g.*, modified storage proteins (U.S. Application Serial No. 10/053,410, filed November 7, 2001); and thioredoxins (U.S. Application Serial No. 10/005,429, filed December 3, 2001)), the disclosures of which are herein incorporated by reference. The polynucleotides of the embodiments can also be stacked with traits desirable for insect, disease or herbicide resistance (*e.g.*, *Bacillus thuringiensis* toxic proteins (U.S. Patent Nos. 5,366,892; 5,747,450; 5,737,514; 5723,756; 5,593,881; Geiser et al (1986) *Gene* 48:109); lectins (Van Damme *et al.* (1994) *Plant Mol. Biol.* 24:825); fumonisin detoxification genes (U.S. Patent No. 5,792,931); avirulence and disease resistance genes (Jones *et al.* (1994) Science 266:789; Martin *et al.* (1993) Science 262:1432; Mindrinos *et al.* (1994) Cell 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS genes, GAT genes such as those disclosed in U.S. Patent Application Publication US2004/0082770, also WO02/36782 and WO03/092360)); and traits desirable for processing or process products such as high oil (*e.g.*, U.S. Patent No. 6,232,529); modified oils (*e.g.*, fatty acid desaturase genes (U.S. Patent No. 5,952,544; WO 94/11516)); modified starches (*e.g.*, ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (SDBE)); and polymers or bioplastics (*e.g.*, U.S. patent No. 5.602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert *et al.* (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)), the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the embodiments with polynucleotides providing agronomic traits such as male sterility (*e.g.*, see U.S. Patent No. 5.583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (*e.g.* WO 99/61619; WO 00/17364; WO 99/25821), the disclosures of which are herein incorporated by reference.

These stacked combinations can be created by any method including but not limited to cross breeding plants by any conventional or TopCross® methodology, or genetic transformation. If the traits are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits

can be used as the target to introduce further traits by subsequent transformation. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference.

The methods of the embodiments involve introducing a polypeptide or polynucleotide into a plant. "Introducing" is intended to mean presenting to the plant the polynucleotide. In some embodiments, the polynucleotide will be presented in such a manner that the sequence gains access to the interior of a cell of the plant, including its potential insertion into the genome of a plant. The methods of the embodiments do not depend on a particular method for introducing a sequence into a plant, only that the polynucleotide gains access to the interior of at least one cell of the plant. Methods for introducing polynucleotides into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods. Polypeptides can also be introduced to a plant in such a manner that they gain access to the interior of the plant cell or remain external to the cell but in close contact with it.

"Stable transformation" is intended to mean that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by the progeny thereof. "Transient transformation" or "transient expression" is intended to mean that a polynucleotide is introduced into the plant and does not integrate into the genome of the plant or a polypeptide is introduced into a plant.

Transformation protocols as well as protocols for introducing polypeptides or polynucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing polypeptides and polynucleotides into plant cells include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,563,055-and 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent Nos. 4,945,050; 5,879,918; 5,886,244; and 5,932,782; Tomes *et al.* (1995) in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe *et al.* (1988) *Biotechnology* 6:923-926); and Lec1 transformation (WO 00/28058). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); U.S. Patent Nos. 5,240,855; 5,322,783 and 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

In specific embodiments, the antipathogenic sequences of the embodiments can be provided to a plant using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the

introduction of the antipathogenic protein or variants and fragments thereof directly into the plant or the introduction of the antipathogenic protein transcript into the plant. Such methods include, for example, microinjection or particle bombardment. See, for example, Crossway *et al.* (1986) *Mol Gen. Genet. 202*:179-185; Nomura *et al.* (1986) *Plant Sci. 44:53-58*; Hepler *et al.* (1994) *Proc. Natl. Acad. Sci. 91*: 2176-2180 and Hush *et al.* (1994) *The Journal of Cell Science 107*:775-784, all of which are herein incorporated by reference. Alternatively, the polynucleotide can be transiently transformed into the plant using techniques known in the art. Such techniques include a viral vector system and the precipitation of the polynucleotide in a manner that precludes subsequent release of the DNA. Thus, the transcription from the particle-bound DNA can occur, but the frequency with which it's released to become integrated into the genome is greatly reduced. Such methods include the use of particles coated with polyethylimine (PEI; Sigma #P3143).

In other embodiments, the polynucleotides of the embodiments may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the embodiments within a viral DNA or RNA molecule. It is recognized that the an antipathogenic polypeptide of the embodiments may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Further, it is recognized that promoters of the embodiments also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing polynucleotides into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367, 5,316,931, and Porta *et al.* (1996) *Molecular Biotechnology 5*:209-221; herein incorporated by reference.

Methods are known in the art for the targeted insertion of a polynucleotide at a specific location in the plant genome. In one embodiment, the insertion of the polynucleotide at a desired genomic location is achieved using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference. Briefly, the polynucleotide of the embodiments can be contained in a transfer cassette flanked by two non-recombinogenic recombination sites. The transfer cassette is introduced into a plant having stably incorporated into its

genome a target site which is flanked by two non-recombinogenic recombination sites that correspond to the sites of the transfer cassette. An appropriate recombinase is provided and the transfer cassette is integrated at the target site. The polynucleotide of interest is thereby integrated at a specific chromosomal position in the plant genome.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting progeny having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the embodiments provide transformed seed (also referred to as "transgenic seed") having a nucleotide construct of the embodiments, for example, an expression cassette of the embodiments, stably incorporated into their genome.

As used herein, the term "plant" includes whole plants, plant cells, plant protoplasts, plant cell tissue cultures from which a maize plant can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, seeds, endosperm, seed coat, leaves, flowers, floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules)branches, fruit, kernels, ears, cobs, husks, stalks, tubers, roots, root tips, anthers, plant tissue (*e.g.* vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like) and progeny of same. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the embodiments, provided that these parts comprise the introduced polynucleotides. The class of plants that can be used in the method of the embodiments is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous.

The methods of the embodiments may be used to induce pathogen resistance or protect from pathogen attack any plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon lycopersicon*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing the embodiments include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis*

nootkatensis). In specific embodiments, plants of the embodiments are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.). In other embodiments, corn and soybean plants are optimal, and in yet other embodiments corn plants are optimal.

Other plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

Antipathogenic compositions, particularly antifungal compositions, are also encompassed by the present invention. Antipathogenic compositions may comprise antipathogenic polypeptides or transformed microorganisms comprising a nucleotide sequence that encodes an antipathogenic polypeptide. The antipathogenic compositions of the invention may be applied to the environment of a plant pathogen, as described herein below, thereby protecting a plant from pathogen attack. Moreover, an antipathogenic composition can be formulated with an acceptable carrier that is, for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granule, a wettable powder, and an emulsifiable concentrate, an aerosol, an impregnated granule, an adjuvant, a coatable paste, and also encapsulations in, for example, polymer substances.

A gene encoding an antipathogenic, particularly nematicidal or antifungal, polypeptide of the embodiments may be introduced into any suitable microbial host according to standard methods in the art. For example, microorganism hosts that are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplana) of one or more crops of interest may be selected. These microorganisms are selected so as to be capable of successfully competing in the particular environment with the wild-type microorganisms, and to provide for stable maintenance and expression of the gene expressing the antipathogenic protein.

Such microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms such as bacteria, *e.g.*, *Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylius, Agrobacterium, Acetobacter, Lactobacillus,*

Arthrobacter, Azotobacter, Leuconostoc, and *Alcaligenes*, fungi, particularly yeast, *e.g.*, *Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula,* and *Aureobasidium.* Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacteria, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus, Clavibacter xyli and Azotobacter vinlandir* and phytosphere yeast species such as *Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces rosues, S. odorus, Kluyveromyces veronae,* and *Aureobasidium pollulans.* Of particular interest are the pigmented microorganisms.

Other illustrative prokaryotes, both Gram-negative and gram-positive, include *Enterobacteriaceae*, such as *Escherichia*, *Erwinia*, *Shigella*, *Salmonella*, *and Proteus*; *Bacillaceae*; *Rhizobiceae*, such as *Rhizobium*; *Spirillaceae*, such as photobacterium, *Zymomonas*, *Serratia*, *Aeromonas*, *Vibrio*, *Desulfovibrio*, *Spirillum; Lactobacillaceae*; *Pseudomonadaceae*, such as *Pseudomonas* and *Acetobacter*; *Azotobacteraceae* and *Nitrobacteraceae.* Among eukaryotes are fungi, such as *Phycomycetes* and *Ascomycetes*, which includes yeast, such as *Saccharomyces* and *Schizosaccharomyces*; and *Basidiomycetes* yeast, such as *Rhodotorula*, *Aureobasidium*, *Sporobolomyces*, and the like.

Microbial host organisms of particular interest include yeast, such as *Rhodotorula spp.*, *Aureobasidium spp.*, *Saccharomyces spp.*, and *Sporobolomyces spp.*, phylloplane organisms such as *Pseudomonas spp.*, *Erwinia spp.,* and *Flavobacterium spp.*, and other such organisms, including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, *Bacillus thuringiensis*, *Escherichia coli*, *Bacillus subtilis*, and the like.

Genes encoding the antipathogenic proteins of the embodiments can be introduced into microorganisms that multiply on plants (epiphytes) to deliver antipathogenic proteins to potential target pathogens. Epiphytes, for example, can be gram-positive or gram-negative bacteria.

Root-colonizing bacteria, for example, can be isolated from the plant of interest by methods known in the art. Specifically, a *Bacillus cereus* strain that colonizes roots can be isolated from roots of a plant (see, for example, Handelsman *et al.* (1991) *Appl. Environ. Microbiol.* 56:713-718). Genes encoding the antifungal

or nematicidal polypeptides of the embodiments can be introduced into a root-colonizing *Bacillus cereus* by standard methods known in the art.

Genes encoding antifungal or nematicidal proteins can be introduced, for example, into the root-colonizing *Bacillus* by means of electrotransformation. Specifically, genes encoding the proteins can be cloned into a shuttle vector, for example, pHT3101 (Lerecius *et al.* (1989) *FEMS Microbiol. Letts.* 60: 211-218. The shuttle vector pHT3101 containing the coding sequence for the particular protein can, for example, be transformed into the root-colonizing *Bacillus* by means of electroporation (Lerecius *et al.* (1989) *FEMS Microbiol. Letts.* 60: 211-218).

Methods are provided for protecting a plant from a pathogen comprising applying an effective amount of an antipathogenic protein or composition of the invention to the environment of the pathogen. "Effective amount" is intended to mean an amount of a protein or composition sufficient to control a pathogen. The antipathogenic proteins and compositions can be applied to the environment of the pathogen by methods known to those of ordinary skill in the art.

The antifungal compositions of the invention may be obtained by the addition of a surface-active agent, an inert carrier, a preservative, a humectant, a feeding stimulant, an attractant, an encapsulating agent, a binder, an emulsifier, a dye, a UV protective, a buffer, a flow agent or fertilizers, micronutrient donors, or other preparations that influence plant growth. One or more agrochemicals including, but not limited to, herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides, acaracides, plant growth regulators, harvest aids, and fertilizers, can be combined with carriers, surfactants or adjuvants customarily employed in the art of formulation or other components to facilitate product handling and application for particular target pathogens. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, *e.g.*, natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders, or fertilizers. The active ingredients of the present invention are normally applied in the form of compositions and can be applied to the crop area, plant, or seed to be treated. For example, the compositions of the present invention may be applied to grain in preparation for or during storage in a grain bin or silo, *etc.* The compositions of the present invention may be applied simultaneously or in succession with other compounds. Methods of applying an active ingredient of the present invention or an agrochemical composition of the

present invention that contains at least one of the antipathogenic proteins, more particularly antifungal proteins, of the present invention include, but are not limited to, foliar application, seed coating, and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pest or pathogen.

Suitable surface-active agents include, but are not limited to, anionic compounds such as a carboxylate of, for example, a metal; carboxylate of a long chain fatty acid; an N-acylsarcosinate; mono or di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty alcohol sulfates such as sodium dodecyl sulfate, sodium octadecyl sulfate or sodium cetyl sulfate; ethoxylated fatty alcohol sulfates; ethoxylated alkylphenol sulfates; lignin sulfonates; petroleum sulfonates; alkyl aryl sulfonates such as alkyl-benzene sulfonates or lower alkylnaphtalene sulfonates, *e.g.*, butyl-naphthalene sulfonate; salts of sulfonated naphthalene-formaldehyde condensates; salts of sulfonated phenol-formaldehyde condensates; more complex sulfonates such as the amide sulfonates, *e.g.*, the sulfonated condensation product of oleic acid and N-methyl taurine; or the dialkyl sulfosuccinates, *e.g.*, the sodium sulfonate or dioctyl succinate. Non-ionic agents include condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, *e.g.*, sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, *e.g.*, polyoxyethylene sorbitar fatty acid esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2,4,7,9-tetraethyl-5-decyn-4,7-diol, or ethoxylated acetylenic glycols. Examples of a cationic surface-active agent include, for instance, an aliphatic mono-, di-, or polyamine such as an acetate, naphthenate or oleate; or oxygen-containing amine such as an amine oxide of polyoxyethylene alkylamine; an amide-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

Examples of inert materials include but are not limited to inorganic minerals such as kaolin, phyllosilicates, carbonates, sulfates, phosphates, or botanical materials such as cork, powdered corncobs, peanut hulls, rice hulls, and walnut shells.

The antipathogenic compositions of the present invention can be in a suitable form for direct application or as a concentrate of primary composition that

requires dilution with a suitable quantity of water or other diluant before application. The concentration of the antipathogenic polypeptide will vary depending upon the nature of the particular formulation, specifically, whether it is a concentrate or to be used directly. The composition contains 1 to 98% of a solid or liquid inert carrier, and 0 to 50%, optimally 0.1 to 50% of a surfactant. These compositions will be administered at the labeled rate for the commercial product, optimally about 0.01 lb-5.0 lb. per acre when in dry form and at about 0.01 pts. - 10 pts. per acre when in liquid form.

In a further embodiment, the compositions, as well as the transformed microorganisms and antipathogenic proteins, of the invention can be treated prior to formulation to prolong the antipathogenic, particularly antifungal, activity when applied to the environment of a target pathogen as long as the pretreatment is not deleterious to the activity. Such treatment can be by chemical and/or physical means as long as the treatment does not deleteriously affect the properties of the composition(s). Examples of chemical reagents include but are not limited to halogenating agents; aldehydes such a formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride; alcohols, such as isopropanol and ethanol; and histological fixatives, such as Bouin's fixative and Helly's fixative (see, for example, Humason (1967) *Animal Tissue Techniques* (W.H. Freeman and Co.).

The antipathogenic compositions of the invention can be applied to the environment of a plant pathogen by, for example, spraying, atomizing, dusting, scattering, coating or pouring, introducing into or on the soil, introducing into irrigation water, by seed treatment or general application or dusting at the time when the pathogen has begun to appear or before the appearance of pathogens as a protective measure. For example, the antipathogenic protein and/or transformed microorganisms of the invention may be mixed with grain to protect the grain during storage. It is generally important to obtain good control of pathogens in the early stages of plant growth, as this is the time when the plant can be most severely damaged. The compositions of the invention can conveniently contain an insecticide if this is thought necessary. In one embodiment of the invention, the composition is applied directly to the soil, at a time of planting, in granular form of a composition of a carrier and dead cells of a *Bacillus* strain or transformed microorganism of the invention. Another embodiment is a granular form of a composition comprising an agrochemical such as, for example, a herbicide, an

insecticide, a fertilizer, an inert carrier, and dead cells of a *Bacillus* strain or transformed microorganism of the invention.

Compositions of the invention find use in protecting plants, seeds, and plant products in a variety of ways. For example, the compositions can be used in a method that involves placing an effective amount of the antipathogenic, more particularly, antifungal, composition in the environment of the pathogen by a procedure selected from the group consisting of spraying, dusting, broadcasting, or seed coating.

Before plant propagation material (fruit, tuber, bulb, corm, grains, seed), but especially seed, is sold as a commercial product, it is customarily treated with a protective coating comprising herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides, or mixtures of several of these preparations, if desired together with further carriers, surfactants, or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal, or animal pests. In order to treat the seed, the protective coating may be applied to the seeds either by impregnating the tubers or grains with a liquid formulation or by coating them with a combined wet or dry formulation. In addition, in special cases, other methods of application to plants are possible, *e.g.*, treatment directed at the buds or the fruit.

The plant seed of the invention comprising a DNA molecule comprising a nucleotide sequence encoding an antipathogenic polypeptide of the invention may be treated with a seed protective coating comprising a seed treatment compound, such as, for example, captan, carboxin, thiram, methalaxyl, pirimiphos-methyl, and others that are commonly used in seed treatment. Alternatively, a seed of the invention comprises a seed protective coating comprising an antipathogenic, more particularly antifungal, composition of the invention is used alone or in combination with one of the seed protective coatings customarily used in seed treatment.

The antifungal polypeptides of the invention can be used for any application including coating surfaces to target microbes. In this manner, the target microbes include human pathogens or microorganisms. Surfaces that might be coated with the antifungal polypeptides of the invention include carpets and sterile medical facilities. Polymer bound polypeptides of the invention may be used to coat surfaces. Methods for incorporating compositions with antimicrobial properties into

polymers are known in the art. See U.S. Patent No. 5,847,047, herein incorporated by reference.

The methods of the embodiments may be effective against a variety of plant fungal pathogens, such as, for example, *Colletotrichum graminocola, Diplodia maydis, Verticillium dahliae, Fusarium graminearum, Fusarium oxysporum* and *Fusarium verticillioides*. Pathogens of the embodiments include nematodes and fungi. Fungal pathogens, include but are not limited to, *Colletotrichum graminicola, Diplodia maydis, Fusarium graminearum,* and *Fusarium verticillioides*. Specific pathogens for the major crops include: Soybeans: *Phytophthora megasperma* fsp. *glycinea*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *sojae (Phomopsis sojae)*, *Diaporthe phaseolorum* var. *caulivora*, *Sclerotium rolfsii*, *Cercospora kikuchii*, *Cercospora sojina*, *Peronospora manshurica*, *Colletotrichum dematium (Colletotichum truncatum)*, *Corynespora cassiicola*, *Septoria glycines*, *Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. *glycinea*, *Xanthomonas campestris* p.v. *phaseoli*, *Microsphaera diffusa*, *Fusarium semitectum*, *Phialophora gregata*, *Glomerella glycines*, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, *Fusarium solani*; Canola: *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Mycosphaerella brassicicola*, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum*, *Alternaria alternata*; Alfalfa: *Clavibacter michiganese subsp. insidiosum*, *Pythium ultimum*, *Pythium irregulare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var. *medicaginis*, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrochila medicaginis*, *Fusarium oxysporum*, *Verticillium albo-atrum, Xanthomonas campestris* p.v. *alfalfae*, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*, *Colletotrichum trifolii*, *Leptosphaerulina briosiana*, *Uromyces striatus*, *Sclerotinia trifoliorum*, *Stagonospora meliloti*, *Stemphylium botryosum*, *Leptotrichila medicaginis*; Wheat: *Pseudomonas syringae* p.v. *atrofaciens*, *Urocystis agropyri*, *Xanthomonas campestris* p.v. *translucens*, *Pseudomonas syringae* p.v. *syringae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Collotetrichum graminicola*, *Erysiphe graminis* f.sp. *tritici*, *Puccinia*

graminis f.sp. *tritici*, *Puccinia recondita* f.sp. *tritici*, *Puccinia striiformis*, *Pyrenophora tritici-repentis*, *Septoria nodorum*, *Septoria tritici*, *Septoria avenae*, *Pseudocercosporella herpotrichoides*, *Rhizoctonia solani*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis* var. *tritici*, *Pythium aphanidermatum*, *Pythium arrhenomanes*, *Pythium ultimum*, *Bipolaris sorokiniana*, *Claviceps purpurea*, *Tilletia tritici*, *Tilletia laevis*, *Ustilago tritici*, *Tilletia indica*, *Rhizoctonia solani*, *Pythium arrhenomannes*, *Pythium gramicola*, *Pythium aphanidermatum*, Sunflower: *Plasmopora halstedii*, *Sclerotinia sclerotiorum*, *Septoria helianthi*, *Phomopsis helianthi*, *Alternaria helianthi*, *Alternaria zinniae*, *Botrytis cinerea*, *Phoma macdonaldii*, *Macrophomina phaseolina*, *Erysiphe cichoracearum*, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus stolonifer*, *Puccinia helianthi*, *Verticillium dahliae*, *Erwinia carotovorum* pv. *carotovora*, *Cephalosporium acremonium*, *Phytophthora cryptogea*, *Albugo tragopogonis*; Corn: *Colletotrichum graminicola*, *Fusarium verticillioides* var. *subglutinans*, *Erwinia stewartii*, *F. verticillioides*, *Gibberella zeae (Fusarium graminearum)*, *Stenocarpella maydi (Diplodia maydis)*, *Pythium irregulare*, *Pythium debaryanum*, *Pythium graminicola*, *Pythium splendens*, *Pythium ultimum*, *Pythium aphanidermatum*, *Aspergillus flavus*, *Bipolaris maydis* O, T *(Cochliobolus heterostrophus)*, *Helminthosporium carbonum* I, II & III *(Cochliobolus carbonum)*, *Exserohilum turcicum* I, II & III, *Helminthosporium pedicellatum*, *Physoderma maydis*, *Phyllosticta maydis*, *Kabatiella maydis*, *Cercospora sorghi*, *Ustilago maydis*, *Puccinia sorghi*, *Puccinia polysora*, *Macrophomina phaseolina*, *Penicillium oxalicum*, *Nigrospora oryzae*, *Cladosporium herbarum*, *Curvularia lunata*, *Curvularia inaequalis*, *Curvularia pallescens*, *Clavibacter michiganense* subsp. *nebraskense*, *Trichoderma viride*, *Claviceps sorghi*, *Pseudomonas avenae*, *Erwinia chrysanthemi* pv. *zea*, *Erwinia carotovora*, *Corn stunt spiroplasma*, *Diplodia macrospora*, *Sclerophthora macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora maydis*, *Peronosclerospora sacchari*, *Sphacelotheca reiliana*, *Physopella zeae*, *Cephalosporium maydis*, *Cephalosporium acremonium*, Sorghum: *Exserohilum turcicum*, *C. sublineolum*, *Cercospora sorghi*, *Gloeocercospora sorghi*, *Ascochyta sorghina*, *Pseudomonas syringae* p.v. *syringae*, *Xanthomonas campestris* p.v. *holcicola*, *Pseudomonas andropogonis*, *Puccinia purpurea*, *Macrophomina phaseolina*, *Perconia circinata*, *Fusarium verticillioides*, *Alternaria alternata*, *Bipolaris sorghicola*, *Helminthosporium sorghicola*, *Curvularia lunata*, *Phoma*

insidiosa, *Pseudomonas avenae (Pseudomonas alboprecipitans)*, *Ramulispora sorghi*, *Ramulispora sorghicola*, *Phyllachara sacchari*, *Sporisorium reilianum (Sphacelotheca reiliana)*, *Sphacelotheca cruenta*, *Sporisorium sorghi*, *Claviceps sorghi*, *Rhizoctonia solani*, *Acremonium strictum*, *Sclerophthona macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Sclerospora graminicola*, *Fusarium graminearum*, *Fusarium oxysporum*, *Pythium arrhenomanes*, *Pythium graminicola*, etc.

The methods of the embodiments may also be effective against a variety of plant nematode pathogens, such as, for example, parasitic nematodes such as root-knot, cyst, and lesion nematodes, including *Heterodera* spp., *Meloidogyne* spp., and *Globodera* spp.; particularly members of the cyst nematodes, including, but not limited to, *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode); *Heterodera avenae* (cereal cyst nematode); *Meloidogyne incognita* (cotton cyst nematode) and *Globodera rostochiensis* and *Globodera pailida* (potato cyst nematodes). Lesion nematodes include *Pratylenchus* spp.

The article "a" and "an" are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element.

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acid sequences are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. The above-defined terms are more fully defined by reference to the specification as a whole.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXPERIMENTAL

Example 1: *C. elegans* feeding bioassays

A number of polypeptides were recombinantly expressed in *E. coli* and then screened in a nematode bioassay.

***C.elegans* feeding bioassays.** Screening was performed in high throughput using *E. coli* expressed material, corresponding to the predicted mature regions of various small peptides. Expression of biologically functional polypeptides involved producing a fusion protein that included a maltose-binding protein (MBP) and a polypeptide of interest and subsequently cleaving the fusion protein at a protease recognition sequence to release the peptide of interest. DNA encoding the polypeptide of interest was fused to the C-terminus of the MelE gene in the *E. coli* expression vector pMAL (New England Biolabs; *see*, Guan *et al.*, *Gene* 67:21-30 (1987); and Maina *et al.*, *Gene* 74:365-73 (1988)). Sequences encoding the cleavage site of proteases Factor Xa or Genenase I were incorporated between the genes of MBP and the polypeptide of interest. A histidine tag was also added to the N-terminus of MBP.

The constructed plasmid vector was transformed into cells of *E. coli* XL-1 Blue and transformants were grown in 2YT medium containing 50 μg/mL carbenicillin to a cell density of OD_{600} = 0.6-0.9. Expression of the fusion protein was induced by addition of IPTG into the culture to a final concentration of 1 mM. Cells were grown for 4-16 hours to saturation before harvesting. Cells were harvested by centrifugation and then lysed with B-PER reagent (Pierce Chemicals, Rockford, IL) to obtain the fraction of soluble proteins. The fusion protein was purified from the cell lysate supernatant utilizing the histidine tag by incubating the cell lysate with Ni-NTA agarose resins for 20 minutes to 1 hour. The resins were washed with Tris buffer to remove all unbound proteins. Two-mercaptoethanol (10 mM) was included in the lysis and washing buffers to allow partial refolding of the proteins. Elution of the bound fusion protein was performed with buffer containing 20-40 mM histidine. To release the polypeptide of interest, the purified fusion protein was incubated with Factor Xa or Genease I (RT, 8-24 hours). The cleaved protein sample was then used in nematode activity assays.

Hit characterization. Approximate IC_{50}'s (in µg/mL) for 4 hits determined from *C. elegans* bioassay using cleaved peptides. Concentrations were determined based on Bradford (BSA standard) and assume 100% cleavage of the MBP-Kistrin fusion.

Kis2	SEQ ID NO:1	15 µg/mL
Kis5	SEQ ID NO:4	75 µg/mL
Kis6	SEQ ID NO:7	150 µg/mL
Kis10	SEQ ID NO:10	30 µg/mL

Example 2: *M. incognita* feeding bioassays in hairy roots and transgenic tobacco

Kistrins in *Astragalus* hairy roots. Two of the polypeptides described in Example 1 (Kis2 (SEQ ID NO: 1) and Kis10 (SEQ ID NO: 10)) were used for *in planta* testing against root knot nematode (*Meloidogyne incognita*). For *in planta* expression, four different gene constructs were prepared. All constructs are in pMAXY 4764 binary vector. A double MMV promoter in this vector drove the expression of the kistrins.

Kis2 is an *Arabidopsis* gene and Kis10 is a *Petunia* gene. The sequence of the full-length cDNA containing an N-terminal extension sequence coding for the pre-pro and signal peptide is available for these two genes in GenBank. A total of four expression cassettes were prepared for *in planta* analysis. The designation of the binary vector constructs is as follows:

pVER 007 – SEQ ID NO:3, encoding SEQ ID NO: 1 – Kis2 (Mature peptide)
pVER 008 – SEG ID NO:13, encoding SEQ ID NO: 2 – Kis2 (Full-length)
pVER 009 – SEQ ID NO:12, encoding SEQ ID NO: 10 – Kis10 (Mature peptide)
pVER 010 – SEQ ID NO:14, encoding SEQ ID NO: 11 – Kis10 (Full-length)

Agrobacterium rhizogenes strain (K599) was transformed with the above constructs. pMAXY 4764 empty vector served as a negative control. *Astragalus sinicus* hypocotyls were co-cultivated with *A.rhizogenes* transformants following standard tissue culture protocols. Transformed hairy roots were regenerated on nutrient media plates containing kanamycin as a plant selectable marker.

After regeneration, the hairy roots were transferred to multiple petri plates for inoculation. *Meloidogyne incognita* stocks are maintained on MiroTom tomato plants in growth rooms. Nematode eggs were harvested from infected plants and hairy roots were inoculated under sterile conditions following standard protocols.

The hairy roots were scored approximately five weeks post-inoculation. Scoring for resistance/susceptibility was performed by extracting and counting five different aliquots of the eggs from each plate. The final data is expressed as percentage of eggs relative to the empty vector control. A summary of the results is presented in Figure 2.

Agrobacterium tumefaciens strain (EHA105) was transformed with the above constructs. pMAXY4764 empty vector served as a negative control. Tobacco (variety Petit Havana) leaf explants were co-cultivated with *Agrobacterium tumefaciens* transformants following standard tissue culture protocols. Ten independent transgenic lines were generated for each construct. For each line of stable transformant, two clones were prepared. Plants were inoculated with *Meloidogyne inocognita* eggs as explained for hairy roots. Scoring for resistance/susceptibility was performed by collecting roots from each plant, extracting eggs and counting the number of eggs as explained for hairy roots. The final data is expressed as percentage of eggs relative to the empty vector control. A summary of the results is presented in Figure 3.

Example 3: Fungal bioassays

The same polypeptides of Example 1 which were recombinantly expressed in *E. coli* were also screened in an anti-fungal bioassay against five selected fungal pathogens. Table 1 illustrates the results of the anti-fungal screening assay.

All fungal strains were grown and maintained on potato dextrose agar (PDA) plates, in a 30 °C incubator. These plates were kept in smaller secondary containers (per fungal strain), with moist paper towels to maintain high humidity. Spores were harvested in a quarter strength of potato dextrose broth (PDB) after about 2 weeks of growth, counted using a hemacytometer, and subsequently stored in small aliquots at -80 °C.

The frozen spores were diluted to the working concentration (determined empirically for each fungal strain), in a quarter strength of PDB, and 50 µL (per well) were added to sterile, flat-bottomed 96-well assay plates. The assay plates were incubated in the humid boxes at room temperature for 5-7 hours to allow the spores to germinate. Serial dilutions of purified, protease-cleaved fusion protein samples were then added to the assay plates, in 50 µL volumes, for a final assay volume of 100 µL per well. The assay plates were allowed to incubate overnight, in

a humid box, at 30 °C. Antifungal activity was scored after 18 to 48 hours, depending upon the strain of fungus.

Table 1
Primary screening
(score: 0= no effect; 1=partial growth inhibition; 2=strong inhibition):

	SEQ ID NO:	*Alternaria brassicicola*	*Fusarium verticillioides*	*Fusarium oxysporum*	*Botrytis cinerea*	*Verticillium dahliae*
Kis2	1	0	0	0	0	0
Kis5	4	0	0	1	0	2
Kis6	7	0	0	0	0	1
Kis10	10	0	0	0	0	0

Example 4: Transformation and Regeneration of Transgenic Maize Plants

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a nucleotide sequence encoding the antipathogenic polypeptide set forth in SEQ ID NO:1 operably linked to a promoter that drives expression in a maize plant cell and a selectable marker (e.g., the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37), which confers resistance to the herbicide Bialaphos). Alternatively, the selectable marker gene is provided on a separate plasmid.
Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

A plasmid vector comprising a nucleotide sequence encoding the antipathogenic polypeptide set forth in SEQ ID NO:1 operably linked to a promoter that drives expression in a maize cell is made. This plasmid DNA plus plasmid DNA containing a selectable marker (e.g., PAT) is precipitated onto 1.1 μm

(average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 µL prepared tungsten particles in water
10 µL (1 µg) DNA in Tris EDTA buffer (1 µg total DNA)
100 µL 2.5 M $CaC1_2$
10 µL 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 mL 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µL 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µL spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/L Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600

pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for fungal resistance.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 mL/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L thiamine HCl, 120.0 g/L sucrose, 1.0 mg/L 2,4-D, and 2.88 g/L L-proline (brought to volume with D-I H_2O following adjustment to pH 5.8 with KOH); 2.0 g/L Gelrite (added after bringing to volume with D-I H_2O); and 8.5 mg/L silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 mL/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L thiamine HCl, 30.0 g/L sucrose, and 2.0 mg/L 2,4-D (brought to volume with D-I H_2O following adjustment to pH 5.8 with KOH); 3.0 g/L Gelrite (added after bringing to volume with D-I H_2O); and 0.85 mg/L silver nitrate and 3.0 mg/L bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 mL/L MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/L thiamine HCL, 0.10 g/L pyridoxine HCL, and 0.40 g/L glycine brought to volume with polished D-I H_2O) (Murashige and Skoog (1962) *Physiol. Plant.* 15:473), 100 mg/L myo-inositol, 0.5 mg/L zeatin, 60 g/L sucrose, and 1.0 mL/L of 0.1 mM abscisic acid (brought to volume with polished D-I H_2O after adjusting to pH 5.6); 3.0 g/L Gelrite (added after bringing to volume with D-I H_2O); and 1.0 mg/L indoleacetic acid and 3.0 mg/L bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 mL/L MS vitamins stock solution (0.100 g/L nicotinic acid, 0.02 g/L thiamine HCL, 0.10 g/L pyridoxine HCL, and 0.40 g/L glycine brought to volume with polished D-I H_2O), 0.1 g/L myo-inositol, and 40.0 g/L sucrose (brought to volume with polished D-I H_2O after adjusting pH to 5.6); and 6 g/L bacto-agar (added after bringing to volume with polished D-I H_2O), sterilized and cooled to 60°C.

Example 5: Agrobacterium-mediated Transformation of Maize and Regeneration of Transgenic Plants

For *Agrobacterium*-mediated transformation of maize with the polynucleotide construct containing SEQ ID NO: 1, the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the polynucleotide construct to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). The immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is performed. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). The immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). The immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium are cultured on solid medium to regenerate the plants.

Example 6: Transformation Of Somatic Soybean Embryo Cultures and Regeneration Of Soybean Plants

The following stock solutions and media are used for transformation and regeneration of soybean plants:

Stock solutions

Sulfate 100 X Stock: 37.0 g $MgSO_4.7H_2O$, 1.69 g $MnSO_4.H_2O$, 0.86 g $ZnSO_4.7H_2O$, 0.0025 g $CuSO_4.5H_2O$.

Halides 100 X Stock: 30.0 g $CaCl_2.2H_2O$, 0.083 g KI, 0.0025 g $CoCl_2.6H_2O$,

P, B, Mo 100X Stock: 18.5 g KH_2PO_4, 0.62 g H_3BO_3, 0.025 g $Na_2MoO_4.2H_2O$

Fe EDTA 100X Stock: 3.724 g Na_2EDTA, 2.784 g $FeSO_4.7H_2O$.

2,4-D Stock: 10 mg/mL.

Vitamin B5 1000X Stock: 10.0 g *myo*-inositol, 0.10 g nicotinic acid, 0.10 g pyridoxine HCl, 1 g thiamine.

Media (per Liter)

SB196: 10 mL of each of the above stock solutions, 1 mL B5 vitamin stock, 0.463 g $(NH_4)_2$ SO_4, 2.83 g KNO_3, 1 mL 2,4-D stock, 1 g asparagine, 10 g sucrose, pH 5.7.

SB103: 1 pk. Murashige & Skoog salts mixture, 1 mL B5 vitamin stock, 750 mg $MgCl_2$ hexahydrate, 60 g maltose, 2 g gelrite, pH 5.7.

SB166: SB103 supplemented with 5 g per liter activated charcoal.

SB71-4: Gamborg's B5 salts (Gibco-BRL catalog No. 21153-028), 1 mL B5 vitamin stock, 30 g sucrose, 5 g TC agar, pH 5.7.

Soybean embryogenic suspension cultures are maintained in 35 mL liquid medium (SB196) on a rotary shaker (150 rpm) at 28°C with fluorescent lights providing a 16 hour day/8 hour night cycle. Cultures are subcultured every 2 weeks by inoculating approximately 35 mg of tissue into 35 mL of fresh liquid media.

Soybean embryogenic suspension cultures are transformed by the method of particle gun bombardment (see Klein *et al.* (1987) *Nature 327*:70-73) using a DuPont Biolistic PDS1000/He instrument.

In particle gun bombardment procedures it is possible to use purified 1) entire plasmid DNA or, 2) DNA fragments containing only the recombinant DNA expression cassette(s) of interest. For every eight bombardment transformations, 30 μl of suspension is prepared containing 1 to 90 picograms (pg) of DNA fragment per base pair of DNA fragment. The recombinant DNA plasmid or fragment used to express the antifungal gene is on a separate recombinant DNA plasmid or fragment from the selectable marker gene. Both recombinant DNA plasmids or fragments are co-precipitated onto gold particles as follows. The DNAs in suspension are added to 50 μL of a 20 - 60 mg/mL 0.6 μm gold particle suspension and then combined with 50 μL $CaCl_2$ (2.5 M) and 20 μL spermidine (0.1 M) The mixture is pulse vortexed 5 times, spun in a microfuge for 10 seconds, and the supernatant removed. The DNA-coated particles are then washed once with 150 μL of 100% ethanol, pulse vortexed and spun in a microfuge again, and resuspended in 85 μL

of anhydrous ethanol. Five μL of the DNA-coated gold particles are then loaded on each macrocarrier disk.

Approximately 150 to 250 mg of two-week-old suspension culture is placed in an empty 60 mm X 15 mm petri plate and the residual liquid is removed from the tissue using a pipette. The tissue is placed about 3.5 inches away from the retaining screen and each plate of tissue is bombarded once. Membrane rupture pressure is set at 650 psi and the chamber is evacuated to -28 inches of Hg. Eighteen plates are bombarded, and, following bombardment, the tissue from each plate is divided between two flasks, placed back into liquid media, and cultured as described above.

Seven days after bombardment, the liquid medium is exchanged with fresh SB196 medium supplemented with 50 mg/mL hygromycin or 100 ng/mL chlorsulfuron, depending on the selectable marker gene used in transformation. The selective medium is refreshed weekly or biweekly. Seven weeks post-bombardment, green, transformed tissue is observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally-propagated, transformed embryogenic suspension cultures. Thus, each new line is treated as independent transformation event. These suspensions can then be maintained as suspensions of embryos clustered in an immature developmental stage through subculture or can be regenerated into whole plants by maturation and germination of individual somatic embryos.

Transformed embryogenic clusters are removed from liquid culture and placed on solid agar medium (SB166) containing no hormones or antibiotics for one week. Embryos are cultured at 26 °C with mixed fluorescent and incandescent lights on a 16 hour day:8 hour night schedule. After one week, the cultures are then transferred to SB103 medium and maintained in the same growth conditions for 3 additional weeks. Prior to transfer from liquid culture to solid medium, tissue from selected lines is assayed by PCR or Southern analysis for the presence of the antifungal gene.

Somatic embryos become suitable for germination after 4 weeks and are then removed from the maturation medium and dried in empty petri dishes for 1 to 5 days. The dried embryos are then planted in SB71-4 medium where they are

allowed to germinate under the same light and germination conditions described above. Germinated embryos are transferred to sterile soil and grown to maturity.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A transgenic plant having stably incorporated into its genome a polynucleotide encoding a polypeptide comprising an amino acid sequence at least 85% identical to SEQ ID NOs: 1, 2, 4, 5, 7, 8, 10 or 11, wherein said plant has improved pathogen resistance to at least one plant pathogen selected from the group consisting of:

a) plant pathogenic fungi; and

b) plant pathogenic nematodes.

2. The plant according to claim 1, wherein said plant is a monocot.

3. The plant according to claim 1, wherein said plant is a dicot.

4. Transformed seed of the plant of claim 1.

5. The plant according to claim 1, wherein said polynucleotide is operably linked to a promoter that drives expression in a cell of said plant, wherein said promoter is selected from the group consisting of:

a) a constitutive promoter;

b) a tissue-specific promoter;

c) a root-specific promoter;

d) an inducible promoter; and

e) a pathogen-inducible promoter.

6. The plant according to claim 1, wherein the polypeptide comprises a signal sequence.

7. The plant according to claim 1, wherein the polypeptide lacks a signal sequence.

8. The plant according to claim 6, wherein the signal sequence is a secretion signal sequence.

9. The plant according to claim 6, wherein the signal sequence is an organelle signal sequence.

10. The plant according to claim 9, wherein the signal sequence is a plastid signal sequence.

11. The plant according to claim 1, wherein said amino acid sequence is the sequence set forth in SEQ ID NO: 1.

12. The plant according to claim 1, wherein said amino acid sequence is the sequence set forth in SEQ ID NO: 4.

13. The plant according to claim 1, wherein said amino acid sequence is the sequence set forth in SEQ ID NO: 7.

14. The plant according to claim 1, wherein said amino acid sequence is the sequence set forth in SEQ ID NO: 10.

15. A method for enhancing resistance of a plant to a pathogen, said method comprising:

(a) stably transforming a plant cell with at least one expression cassette comprising a nucleotide sequence operably linked to a promoter that drives expression in a cell of said plant, wherein said nucleotide sequence encodes a polypeptide having at least 85% sequence identity to SEQ ID NOs: 1, 2, 4, 5, 7, 8, 10 or 11, and further wherein said polypeptide has activity against at least one pathogen selected from the group consisting of:

i. plant pathogenic fungi; and

ii. plant pathogenic nematodes; and

(b) regenerating a transformed plant from said plant cell, wherein the level of resistance to said pathogen in said plant is increased in comparison to a plant that does not comprise said expression cassette.

16. The method of claim 15, wherein said pathogen is a plant pathogenic fungus.

17. The method of claim 15, wherein said pathogen is a plant pathogenic nematode.

18. The method of claim 15, wherein said fungus is *Fusarium oxysporum*.

19. The method of claim 15, wherein said fungus is *Verticillium dahliae*.

20. The method of claim 15, wherein said nematode is *Meloidogyne incognita*.

21. The method of claim 15, wherein said nucleotide sequence encodes the polypeptide set forth in SEQ ID NO: 1.

22. The method of claim 15, wherein said nucleotide sequence encodes the polypeptide set forth in SEQ ID NO: 4.

23. The method of claim 5, wherein said nucleotide sequence encodes the polypeptide set forth in SEQ ID NO: 7.

24. The method of claim 15, wherein said nucleotide sequence encodes the polypeptide set forth in SEQ ID NO: 10.

25. The method of claim 15, wherein said promoter is selected from the group consisting of:

b) a constitutive promoter;

b) a tissue-specific promoter;

c) a root-specific promoter;

d) an inducible promoter; and

e) a pathogen-inducible promoter.

26. The method of claim 15, wherein the polypeptide comprises a signal sequence.

27. The method of claim 15, wherein the polypeptide lacks a signal sequence.

28. The method of claim 26, wherein the signal sequence is a secretion signal sequence.

29. The method of claim 26, wherein the signal sequence is an organelle signal sequence.

29. The method of claim 26, wherein the signal sequence is a plastid signal sequence.

30. A method for enhancing resistance of a plant to a pathogen, said method comprising:

(a) stably transforming a plant cell with at least one expression cassette comprising a nucleotide sequence operably linked to a promoter that drives expression in a cell of said plant, wherein said nucleotide sequence has at least 85% sequence identity to SEQ ID NOs:3, 6, 9, 12, 13 or 14, and further wherein said nucleotide sequence encodes a polypeptide having activity against at least one pathogen selected from the group consisting of:

i. plant pathogenic fungi; and

ii. plant pathogenic nematodes; and

(b) regenerating a transformed plant from said plant cell, wherein the level of resistance to said pathogen in said plant is increased in comparison to a plant that does not comprise said expression cassette.

31. The method of claim 30, wherein the promoter is selected from the group consisting of:

a) a constitutive promoter;
b) a tissue-specific promoter;
c) a root-specific promoter;
d) an inducible promoter; and
e) a pathogen-inducible promoter.

32. The method of claim 30, wherein the polypeptide comprises a signal sequence.

33. The method of claim 30, wherein the polypeptide lacks a signal sequence.

34. The method of claim 32, wherein the signal sequence is a secretion signal sequence.

35. The method of claim 32,wherein the signal sequence is an organelle signal sequence.

36. The method of claim 32, wherein the signal sequence is a plastid signal sequence.

37. The method of claim 30, wherein the nucleotide sequence is SEQ ID NO:3.

38. The method of claim 30, wherein the nucleotide sequence is SEQ ID NO:6.

39. The method of claim 30, wherein the nucleotide sequence is SEQ ID NO:9.

40. The method of claim 30, wherein the nucleotide sequence is SEQ ID NO:12.

41. The method of claim 30, wherein the nucleotide sequence is SEQ ID NO:13.

42. The method of claim 30, wherein the nucleotide sequence is SEQ ID NO:14.

43. A transgenic plant having stably incorporated into its genome a polynucleotide sequence at least 85% identical to SEQ ID NOs: 3, 6, 9, 12, 13, or 14, wherein said polynucleotide sequence encodes a polypeptide with activity against plant pathogens, further wherein said plant has improved pathogen resistance to at least one plant pathogen selected from the group consisting of:

a) plant pathogenic fungi; and
b) plant pathogenic nematodes.

44. The plant according to claim 43, wherein said plant is a monocot.

45. The plant according to claim 43, wherein said plant is a dicot.

46. Transformed seed of the plant of claim 43.

47. The plant according to claim 43, wherein said polynucleotide is operably linked to a promoter that drives expression in a cell of said plant, wherein said promoter is selected from the group consisting of:

c) a constitutive promoter;

b) a tissue-specific promoter;

c) a root-specific promoter;

d) an inducible promoter; and

e) a pathogen-inducible promoter.

48. The plant according to claim 43, wherein the polypeptide comprises a signal sequence.

49. The plant according to claim 43, wherein the polypeptide lacks a signal sequence.

50. The plant according to claim 48, wherein the signal sequence is a secretion signal sequence.

51. The plant according to claim 48, wherein the signal sequence is an organelle signal sequence.

52. The plant according to claim 48, wherein the signal sequence is a plastid signal sequence.

53. The plant according to claim 43, wherein said polynucleotide sequence is the sequence set forth in SEQ ID NO: 3.

54. The plant according to claim 43, wherein said polynucleotide sequence is the sequence set forth in SEQ ID NO: 6.

55. The plant according to claim 43, wherein said polynucleotide sequence is the sequence set forth in SEQ ID NO: 9.

56. The plant according to claim 43, wherein said polynucleotide sequence is the sequence set forth in SEQ ID NO:12.

57. The plant according to claim 43, wherein said polynucleotide sequence is the sequence set forth in SEQ ID NO:13.

58. The plant according to claim 43, wherein said polynucleotide sequence is the sequence set forth in SEQ ID NO:14.

Figure 1

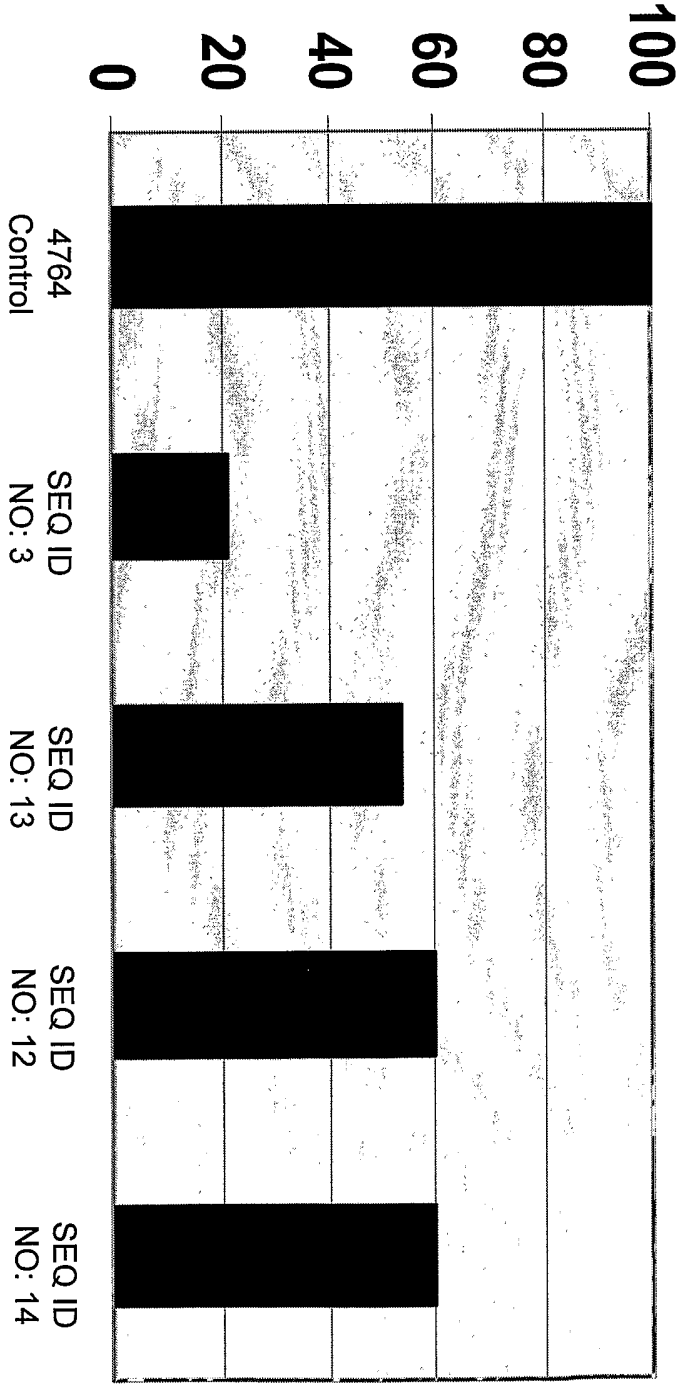
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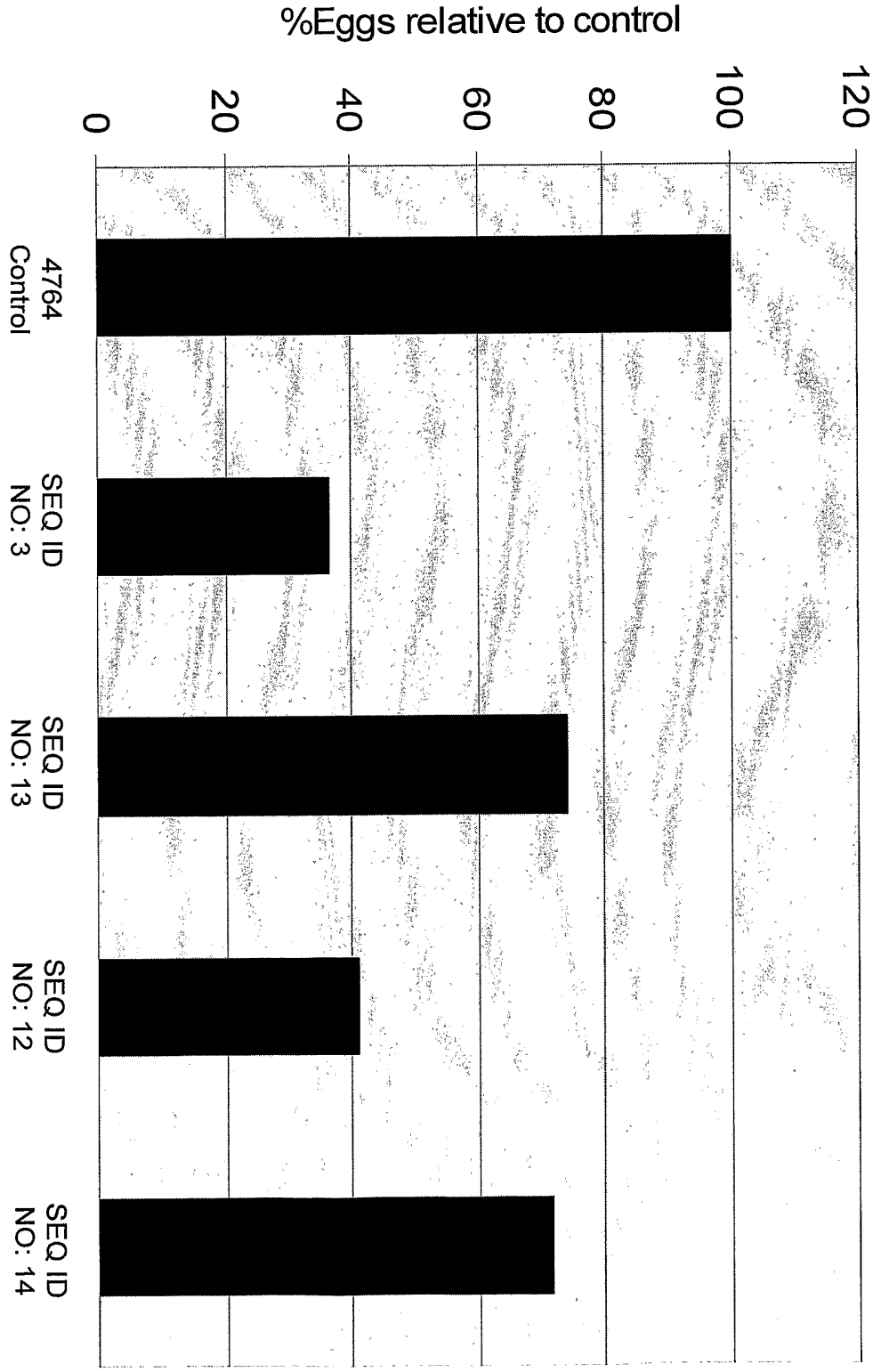
Figure 2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<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> optimized for expression in E. coli

<221> misc_feature
<222> (0)...(0)
<223> Sequence encoding SEQ ID NO:1 as expressed in E.
coli for C. elegans bioassays

<400> 3
ggcaaactga aaccgcaaca gtgtaactct aaatgcagct tccgttgctc cgcaactagc 60
cataagaagc cgtgcatgtt cttttgcctg aaatgttgta aaaagtgcct gtgcgtaccg 120
ccgggcacct tcggcaataa gcagacgtgt ccgtgctaca acaactggaa aactaaagaa 180
ggtcgcccga aatgtcca 198

<210> 4
<211> 66
<212> PRT
<213> Picea mariana

<220>
<221> PEPTIDE
<222> (0)...(0)
<223> Kis5 mature peptide

<400> 4
Gly Ser Leu Arg Pro Ser Glu Cys Gly Gln Arg Cys Ser Tyr Arg Cys
1 5 10 15
Ser Ala Thr Ser His Lys Lys Pro Cys Met Phe Phe Cys Gln Lys Cys
20 25 30
Cys Ala Lys Cys Leu Cys Val Pro Pro Gly Thr Phe Gly Asn Lys Gln
35 40 45
Val Cys Pro Cys Tyr Asn Asn Trp Lys Thr Gln Gln Gly Gly Pro Lys
50 55 60
Cys Pro
65

<210> 5
<211> 110
<212> PRT
<213> Picea mariana

<220>
<221> PEPTIDE
<222> (0)...(0)
<223> AAC32128 (Kis5)

<400> 5
Met Ala Arg Leu Gln Ser Phe Ala Val Leu Leu Ile Thr Ile Phe Ala
1 5 10 15
Leu Phe Ile Trp Asn Ile Glu Ala Ala Leu Pro His Ser Asn Val Asp
20 25 30
Pro Phe Met Glu Gln Lys Gln Gly Gln Tyr Gly Glu Gly Ser Leu Arg
35 40 45

Pro Ser Glu Cys Gly Gln Arg Cys Ser Tyr Arg Cys Ser Ala Thr Ser
50 55 60
His Lys Lys Pro Cys Met Phe Phe Cys Gln Lys Cys Cys Ala Lys Cys
65 70 75 80
Leu Cys Val Pro Pro Gly Thr Phe Gly Asn Lys Gln Val Cys Pro Cys
85 90 95
Tyr Asn Asn Trp Lys Thr Gln Gln Gly Gly Pro Lys Cys Pro
100 105 110

<210> 6
<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> optimized for expression in E. coli

<221> misc_feature
<222> (0)...(0)
<223> Sequence encoding SEQ ID NO:4 as expressed in E.
coli for C. elegans bioassays

<400> 6
ggctctctgc gtccgtctga atgtggtcag cgttgcagct accgttgctc cgcaactagc 60
cataagaagc cgtgcatgtt cttttgccaa aaatgttgtg caaagtgcct gtgcgtaccg 120
ccgggcacct tcggcaataa gcaggtttgt ccgtgctaca acaactggaa aactcagcag 180
ggtggtccga aatgtcca 198

<210> 7
<211> 66
<212> PRT
<213> Arabidopsis thaliana

<220>
<221> PEPTIDE
<222> (0)...(0)
<223> Kis6 mature peptide

<400> 7
Gly Tyr Ala Lys Lys Ile Asp Cys Gly Ser Ala Cys Val Ala Arg Cys
1 5 10 15
Arg Leu Ser Arg Arg Pro Arg Leu Cys His Arg Ala Cys Gly Thr Cys
20 25 30
Cys Tyr Arg Cys Asn Cys Val Pro Pro Gly Thr Tyr Gly Asn Tyr Asp
35 40 45
Lys Cys Gln Cys Tyr Ala Ser Leu Thr Thr His Gly Gly Arg Arg Lys
50 55 60
Cys Pro
65

<210> 8
<211> 98
<212> PRT
<213> Arabidopsis thaliana

<220>
<221> PEPTIDE
<222> (0)...(0)

<223> S60229 (Kis6)

<400> 8
Met Ala Ile Ser Lys Ala Leu Ile Ala Ser Leu Leu Ile Ser Leu Leu
1 5 10 15
Val Leu Gln Leu Val Gln Ala Asp Val Glu Asn Ser Gln Lys Lys Asn
20 25 30
Gly Tyr Ala Lys Lys Ile Asp Cys Gly Ser Ala Cys Val Ala Arg Cys
35 40 45
Arg Leu Ser Arg Arg Pro Arg Leu Cys His Arg Ala Cys Gly Thr Cys
50 55 60
Cys Tyr Arg Cys Asn Cys Val Pro Pro Gly Thr Tyr Gly Asn Tyr Asp
65 70 75 80
Lys Cys Gln Cys Tyr Ala Ser Leu Thr Thr His Gly Gly Arg Arg Lys
85 90 95
Cys Pro

<210> 9
<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> optimized for expression in E. coli.

<221> misc_feature
<222> (0)...(0)
<223> Sequence encoding SEQ ID NO:7 as expressed in E.
coli for C. elegans bioassays

<400> 9
ggctacgcaa aaaaaattga ttgtggttct gcttgcgttg cgcgttgccg tctgtctcgt 60
cgcccacgtc tgtgccaccg cgcctgcggc acttgttgtt accgctgcaa ctgcgtaccg 120
ccgggcacct atggcaatta cgataaatgt cagtgctacg ccagcctgac cactcacggc 180
ggtcgccgca aatgtcca 198

<210> 10
<211> 66
<212> PRT
<213> Petunia x hybrida

<220>
<221> PEPTIDE
<222> (0)...(0)
<223> Kis10 mature peptide

<400> 10
Gly Ser Leu Lys Pro Ser Gln Cys Leu Pro Gln Cys Thr Arg Arg Cys
1 5 10 15
Ser Gln Thr Gln Tyr His Asn Ala Cys Met Leu Phe Cys Gln Lys Cys
20 25 30
Cys Asn Lys Cys Leu Cys Val Pro Pro Gly Phe Tyr Gly Asn Lys Gly
35 40 45
Val Cys Pro Cys Tyr Asn Asn Trp Lys Thr Lys Glu Gly Gly Pro Lys
50 55 60
Cys Pro
65

<210> 11
<211> 105
<212> PRT
<213> Petunia x hybrida

<220>
<221> PEPTIDE
<222> (0)...(0)
<223> CAD10105 (Kis10)

<400> 11
Met Ala Lys Leu Val Pro Ile Phe Leu Leu Ala Leu Phe Val Ile Ser
1 5 10 15
Met Phe Ala Thr Thr Val Leu Ala Ser His Asp Pro Lys Arg Gly His
20 25 30
His His Lys Gly Tyr Gly Pro Gly Ser Leu Lys Pro Ser Gln Cys Leu
35 40 45
Pro Gln Cys Thr Arg Arg Cys Ser Gln Thr Gln Tyr His Asn Ala Cys
50 55 60
Met Leu Phe Cys Gln Lys Cys Cys Asn Lys Cys Leu Cys Val Pro Pro
65 70 75 80
Gly Phe Tyr Gly Asn Lys Gly Val Cys Pro Cys Tyr Asn Asn Trp Lys
85 90 95
Thr Lys Glu Gly Gly Pro Lys Cys Pro
100 105

<210> 12
<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> optimized for expression in E. coli

<221> misc_feature
<222> (0)...(0)
<223> Sequence encoding SEQ ID NO:10 as expressed in E.
coli for C. elegans bioassays

<400> 12
ggctctctga aaccgtctca gtgtctgcca cagtgcaccc gtcgttgctc ccaaactcaa 60
taccataacg catgcatgct gttttgccaa aaatgttgta acaagtgcct gtgcgtaccg 120
ccgggcttct atggcaataa gggcgtttgt ccgtgctaca acaactggaa aactaaagaa 180
ggtggtccga aatgtcca 198

<210> 13
<211> 294
<212> DNA
<213> Artificial Sequence

<220>
<223> optimized for expression in E. coli

<221> misc_difference
<222> (0)...(0)
<223> Sequence encoding SEQ ID NO:2 as expressed in
plant hairy root for anti-nematode bioassays

<400> 13

atggcgaatt gtatcagaag aaatgctctt ttcttcttga ctcttctctt tttattgtca 60
gtctccaacc tcgttcaggc tgctcgtggt ggtggcaaac tgaaaccgca acagtgtaac 120
tctaaatgca gcttccgttg ctccgcaact agccataaga agccgtgcat gttcttttgc 180
ctgaaatgtt gtaaaaagtg cctgtgcgta ccgccgggca ccttcggcaa taagcagacg 240
tgtccgtgct acaacaactg gaaaactaaa gaaggtcgcc cgaaatgtcc ataa 294

<210> 14
<211> 318
<212> DNA
<213> Artificial Sequence

<220>
<223> optimized for expression in E. coli

<221> misc_feature
<222> (0)...(0)
<223> Sequence encoding SEQ ID NO:11 as expressed in
plant hairy root for anti-nematode bioassays

<400> 14
atggccaagc ttgttcccat ttttcttttg gccctatttg tcatctccat gtttgctacc 60
acagttcttg cttcacatga tccaaagcgc gggcaccatc ataaagggta tggaccaggc 120
tctctgaaac cgtctcagtg tctgccacag tgcacccgtc gttgctccca aactcaatac 180
cataacgcat gcatgctgtt ttgccaaaaa tgttgtaaca agtgcctgtg cgtaccgccg 240
ggcttctatg gcaataaggg cgtttgtccg tgctacaaca actggaaaac taaagaaggt 300
ggtccgaaat gtccataa 318